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TECHNICAL REPORT

3D printed scaffold to monitor hanging drops on inverted microscopes

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Abstract:

Growing cells in 3D cell culture became a major field in cell biology over the last decade. Cells growing in a dimensionally unrestricted space spontaneously form organoids or spheroids that express cellular behavior different from cells growing on a plane surface. However, monitoring three dimensional spheroids on standard inverted microscopes proved difficult since hanging drops are often outside the focus range. Here I present the design and production of a cover slip tray by conventional additive printing (3D printing) able to fit into a single well of a six-well plate to enable the observation of hanging drop spheroids using inverted microscopes.



Introduction:

Over the last years cell research on three dimensionally grown organoids or spheroids vastly gained popularity (1, 2). Cells grown in such structures behave differently in terms of metabolism and differentiation to cells grown in conventional 2D cell culture techniques and are believed to act closer to the *in situ* situation of the cells in an organ or tissue (3).

Several methods were established over the years to generate 3D grown spheroids. One approach is to grow cells in Matrigel, a hyper viscous liquid that blocks cells from migrating away from each other and so induces the formation of cell-cell contacts, independent of a solid surface. However, by the hyper viscous consistence of Matrigel it usually happens, that the cell spheroids form hollow balls, since they interpret the Matrigel as an extracellular matrix and the inner side of the spheroid as a liquid surface (4). To avoid this effect, a more simple approach is to grow cells in hanging drops. This method uses the forces of gravity to collect single cells at the lowest point of a droplet mounted to the lid of a cell culture vessel (5). There the cells get in contact to each other and start spontaneously to form cell-cell aggregates. Cells that fail to attach to other cells undergo cell death by anoikis (6). Drop size used range from 5 μ l up to 50 μ l and the cell number seeded in this drop can vary from a few hundred up to 100.000.

One important aspect of cell biology is the visual inspection of cells growing and interacting to other cells. Nowadays this is accomplished by highly specialized microscopes that mostly appear in the inverted phenotype to establish a close working distance between objective and cells without the objective submersing in cell culture medium and putting the cells at risk of contamination (7). Thus inverted microscopes are perfectly adapted to monitor cells grown in standard 2D cell culture conditions and most modern cell culture laboratories are equipped with those microscopes. However, the advent of 3D cell culture techniques brings this established technique to a new challenge. The location of the cells is not anymore at the lowest position of the cell culture vessel and this makes it difficult for the microscope to find the optimal focus plane in a germ free environment.

Here I present the design and construction of an easy to produce cover slip tray that allows the growth of hanging drop spheroids on coverslips in the sterile environment of a 6 well plate. Using this tray the formation of spheroids can be monitored up to several days, without touching or manipulating the spheroid.



Material and Methods:

Design software: The cover slip tray was designed using FreeCAD software (www.freecad.com) and exported as STL (STereoLithography) files to ideaMaker 3.3.0 software for printing. Final versions of the STL files are available as Supplementary material.

3D Printing: Polylactic acid (PLA) filament was purchased from filamentworld.de. The model was produced using a Pro2 3D Printer purchased from Raise3D (California, USA). All printing parameters are shown in Table 1.

Table 1: Settings of the 3D Printer

Printer	Pro2 3D Printer N2, Raise3D (California, USA)
Filament	PLA
Supports	No
Raft	yes
Infill (%)	100
Shells	2
Layer height (mm)	0.1
Extruder temperature (°C)	230
Build plate temperature (°C)	n.a.
Extruder speed while extruding (mm/s)	50 (10-150)
Extruder speed while travelling (mm/s)	180 (150-300)
Model weight (g)	0.94
Print time (hours, approx.)	0.2

Preparation of the Cover Slip Tray: After printing, the tray was cleaned from loose-ends PLA hairlines with forceps or an open flame. Before fixing the circular 12 mm cover slip (#01-0012/1, Langenbrink, Emmendingen, Germany) on the tray a circle with a liquid blocker (*e.g.* Pap Pen, #MKP1, Kisker, Steinfurt, Germany) with a diameter of ~5 mm was drawn to create a hydrophobic barrier to avoid disintegration of the hanging drop by potential vibrations of the



cell culture incubator. The cover slip was then fixed with a small drop of glue to the cover slip tray.

Cell culture: mIMCD3 cells were grown exponentially using 2D cell culture in standard cell culture conditions. Cells were kept in DMEM supplemented with 10% FCS and 1% Pen/Strep. To grow cells in spheroids, a confluent plate of cells was washed with PBS, cells were trypsinised, and after stopping digestion with an excess of DMEM (with supplements), cells were diluted to a concentration of 1×10^6 cells/ml. After vortexing, a drop of 30 μ l cell suspension was carefully put on the coverslip inside the circle of liquid blocker that was previously mounted on the cover slip tray (see above). Using forceps the tray was then inverted and placed into a well of a sterile 6 well plate. To avoid evaporation of the droplet, 200 μ L of PBS was filled into the circular rill of the cover slip tray and the 6 well plate was then transferred to either immediate monitoring in a microscope (see below) or an incubator (37°C, 5% CO₂) to grow the spheroid.

Microscopy and image acquisition: The 6 well plate containing the cover slip tray was placed on an inverted Observer.Z1 microscope (Zeiss, Jena, Germany) and a 20x or 40x Plan-Neofluar lens (NA: 0.4 and 0.6). Images and videos were acquired using the ZEN software. Videos are composites of individual images captured all 120 seconds. Only minor adjustments of brightness and contrast were applied, which in no case altered the original appearance of the images.

Results and Discussion:

One major disadvantage of the hanging drop approach is, that cell culture plates usually have a height of >1 cm, a distance too large to be covered by conventional inverted cell culture microscopes. To bring the hanging drop containing the spheroid closer to the objective of the microscope I developed a cover slip tray that can be put into a single well of a 6 well plate (Fig. 1a-A). It was designed using the freeware freeCAD and manufactured by additive printing in a commercial available 3D Printer (Fig 1b and c). The cover slip tray consists of a spacer ring in the middle that dictates the distance to the objective. This distance can be easily varied by cutting away parts of the plastic. On the hole in the middle a circular coverslip (12 mm diameter) is mounted (Fig 1a-B), that holds a ring of liquid blocker (optimal diameter 5 mm, Fig 1a-E). Inside the ring of liquid blocker the drop containing the cells can be pipetted (Fig 1a-



C). Now, the cover slip tray is carefully inverted using forceps and placed into a 6 well plate. To avoid evaporation of the drop, some PBS is filled in the groove around the coverslip (Fig a-D). After turning the cover slip tray with the mounted drop, cells settle at the lowest point after a few minutes (Fig 1a-C, purple dot) and assembly of the spheroid can be monitored immediately using an inverted microscope (Fig 1d).

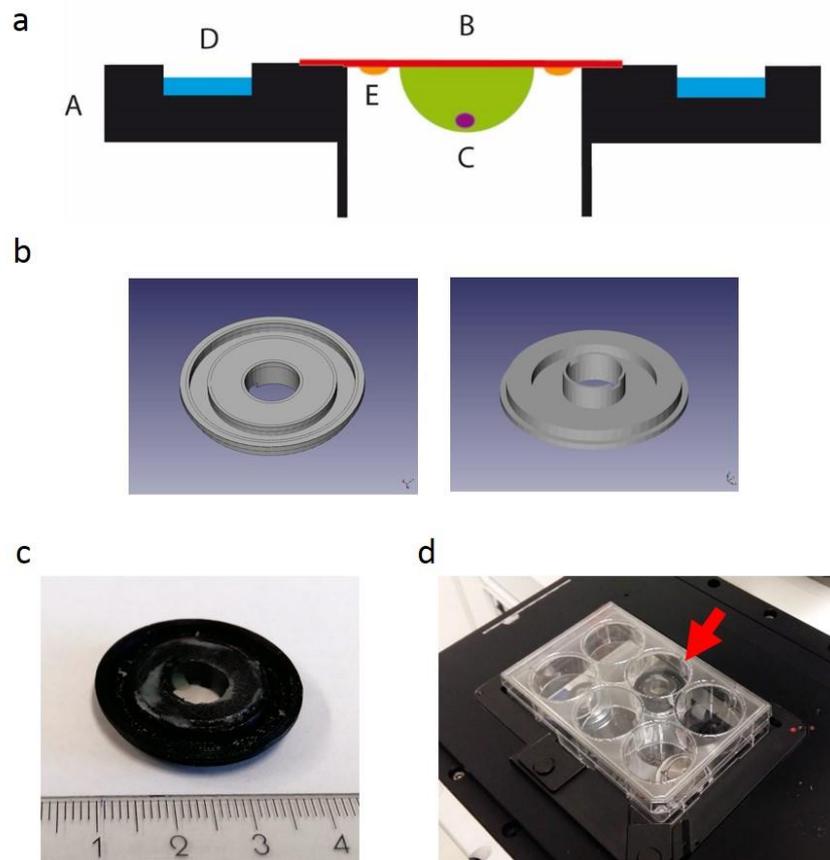


Figure 1: Design and use of a cover slip tray to visualize hanging drops using an inverted microscope. Schematic transection of the cover slip tray (see for detailed description in the text) (a). Axonometric views as seen from top (left) and bottom (right) of the design of the cover slip tray as drawn in freeCAD software (b). Actual product produced by additive printing using a 3D printer (c). Use of the cover slip tray in a 6 well plate (red arrow) on the microscope table to maintain a sterile environment (d).

Twenty-four hours after seeding a spheroid consisting of 30.000 cells the assembled cell clot can be seen with the naked eye. When observed under 20x magnification it can clearly be visualized that the spheroid consists of identical cells that are in tight contact to each other (Fig



2a). Performing time lapse microscopy on the spheroid with a timeframe of 120 seconds one clearly can see, that the spheroid is an amorph collection of individual cells that still migrate within the cell assembly ([Supplementary Video 1](#)). Furthermore, the spheroid induces a current in the medium that is visualized by the clot of dead cells being transported along the spheroid border in the right upper side. This current might be induced by the primary cilia mIMCD3 cells develop once they undergo confluency induced differentiation (8).

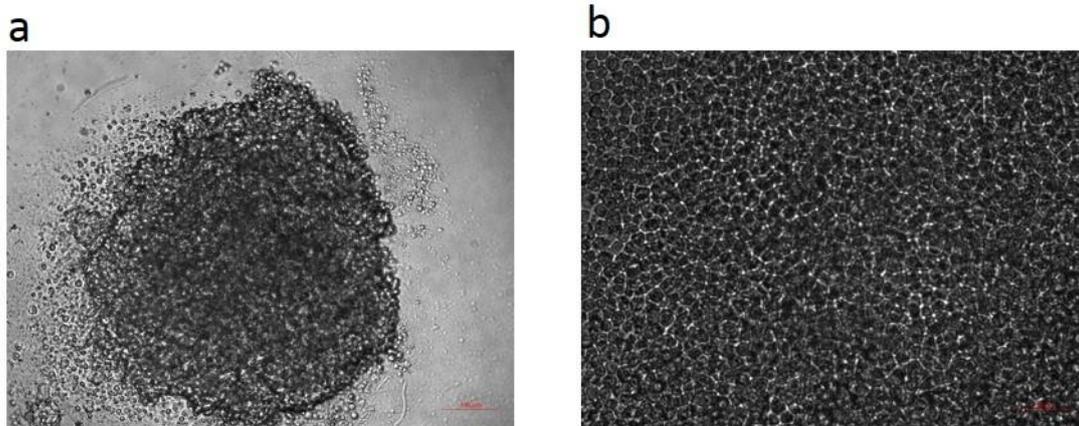


Figure 2: Stills from movies of spheroid formation captured with an inverted microscope using the cover slip tray. Spheroid is clearly visible 24 hours after seeding individualized cells, 20x magnification (a). Individual cells migrate to the lowest point of the hanging drop and start to get in contact to each other approximately 10 minutes after seeding, 40x magnification (b). Size bars represent 100 μm (a) and 50 μm (b).

Imaging the cells right after seeding shows the first steps of the spheroid assembly that will happen the following hours. The cells sink along the gravitational axis and collect at the lowest position of the droplet (Fig 2b and [Supplementary Video 2](#)). Interestingly, however, cells do not stop moving when settling at the water-air border but rather converge towards each other and start to form multi layered cell assemblies. This almost immediate formation of a vertical layering may explain the spheroidal shape of the final cell assembly. Since the cells have no possibility to get any physical grip on the droplet surface (as they would in Matrigel) the only hold they find is their neighboring cells. A cell can establish many contacts to other cells. Doing so, a spheroidal object automatically forms keeping the net mechanical forces within the object as small as possible.



Future experiments will include life cell imaging of extracellular protein interactions to understand, when and how cells start to form cell-cell contacts mediated by surface proteins such as cadherins and integrins. Interrupting such protein interactions by drugs such as the fungal secondary metabolite gliotoxin will further help to understand the behavior of cells when proper protein-protein interaction cannot be established (6).

In summary, I present here an easy to produce a cover slip tray to enable the investigation of hanging drop spheroid formation in 6 well plates using standard inverted microscopes.

Abbreviations:

DMEM: Dulbecco's modified Eagle's medium, PBS: Phosphate buffered saline, Pen/Strep: Penicillin and Streptomycin, PLA: Polylactic acid;

Conflict of Interest:

The author declares no conflict of interest.

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